Separation of β-Lactoglobulin from Other Milk Serum Proteins by Trichloroacetic Acid

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Abstract

β-Lactoglobulin can be easily separated from milk because it is the whey protein most resistant to precipitation by trichloroacetic acid. After removing casein from milk by acid precipitation, the residual whey is made up to contain 3% trichloroacetic acid. All proteins other than β-lactoglobulin precipitate and can be filtered. The filtrate is concentrated by negative pressure dialysis, dialyzed free of low molecular weight materials, and lyophilized. The recovered β-lactoglobulin had a specific rotation of −27.8 at 575 mμ, pH 4.75, an intrinsic viscosity of 4.1, and an electrophoretic mobility of -5.65×10^{-5} $cm^2/v/sec$ in veronal buffer pH 8.6, 0.1 ionic strength. It is monophoretic in this buffer. These values agree closely with those obtained in similar analyses of a commercially available sample of 3× crystallized β-lactoglobulin prepared by salt fractionation.

Palmer, in 1934, reported the isolation of β-lactoglobulin from milk and the crystallization of the product (10). Since that time many improvements in the basic methodology of fractionation have been published (1, 9). In spite of this, isolation of the protein remains a time-consuming process.

During a part of our long-term study of the effect of dairy product manufacture on the properties of milk components, it was noted that, of the proteins in milk whey, β-lactoglobulin is the most resistant to the precipitating action of trichloroacetic acid (TCA).

This paper describes those observations and presents a simplified procedure for isolation of β-lactoglobulin, along with some of the physical properties of the product obtained.

Materials and Methods [1]

Materials. Samples of bovine serum albumin, γ-globulin, α-lactalbumin, and β-lactoglobulin were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. β-Lactoglobulin was a typical product obtained by the salt fractionation technique.

Veronal buffer having a pH of 8.59 at 24 C and 9.12 at 1.2 C was made with 0.5 M sodium diethyl barbituric acid, 0.01 M barbituric acid, and 0.05 M NaCl.

Acetate buffer, pH 4.75 at 25 C, was made with 0.05 M sodium acetate and 0.05 M acetic acid.

Phosphate buffer, pH 7.10 at 25 C, was made with 0.01 M total phosphate and 0.14 M NaCl.

Reagent grade trichloroacetic acid (TCA) was purchased from Baker Chemical Company, Muirkirk, Maryland.

Skimmilk was obtained by separating fresh whole milk produced by herds at the Agricultural Research Center, Beltsville, Maryland.

Procedures. Determination of solubility of bovine proteins in solutions of varying TCA content. Solutions of the individual pure bovine whey and blood proteins were made up by dissolving 0.5 g protein in 100 ml of pH 7.10 phosphate buffer. To 9-ml aliquots of each protein solution 1 ml of TCA solution was added. The concentration of TCA in the added aliquot was so adjusted as to make the TCA concentration in the final mixtures range progressively from 0.5 to 4.0% in 0.5% increments. After standing for 30 min the solutions were cleared of precipitate by centrifugation in a Servall centrifuge. The optical density of the clear supernatant was immediately measured at 278 mμ. The per cent protein not precipitated was calculated by dividing the optical density of the supernatant by that of a 9-ml aliquot brought up to volume by adding 1 ml buffer and multiplying by 100.

The selective precipitating action of TCA in milk whey was studied by first removing casein from skimmilk by adding M HCl acid to pH 4.7 and filtering off the curd. To 100-ml aliquots of the resultant acid whey, sufficient 40% TCA solution was added to make up solutions containing 1, 2, and 3% TCA. After standing 30 min the solutions were cleared of precipitate by low speed centrifugation. The

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[1] Reference to certain products or companies does not imply endorsement by the Department over others not mentioned.

clear supernatants were dialyzed with frequent stirring against frequent changes of distilled water until free of chloride ion. They were then lyophilized. The protein composition of the powders was determined by electrophoresis in polyacrylamide gel (PAG).

Isolation of β-lactoglobulin by use of TCA. The pH of four liters of 38 C skimmilk was adjusted to 4.7 by adding approximately 50 ml M HCl per liter of milk. The precipitated casein was removed by filtration through Miracloth (Calbiochem, Los Angeles, California). Approximately 3.8 liters of acid whey were recovered. To this, 130 g of TCA dissolved in a minimum amount of water was slowly added with continuous stirring. After addition of TCA was completed, the solution was allowed to stand at room temperature for 30 min and then cleared of precipitate by centrifugation in a Servall centrifuge. The clear supernatant was concentrated to 400 ml by using negative pressure dialysis at 4 C for approximately 48 hr.

From this point, two procedures were devised to isolate the residual β-lactoglobulin in the concentrated whey.

(a) Saturated ammonium sulfate solution was added to the concentrated whey to produce 0.4 saturation with respect to ammonium sulfate. The slight precipitate which appeared at this point was removed by centrifugation, and solid ammonium sulfate then added to saturate the cleared solution. After standing overnight at room temperature, the precipitate was removed from the solution by centrifugation, resuspended in saturated ammonium sulfate, again recovered by centrifugation, and finally dissolved in 400 ml of distilled water. The solution was dialyzed against distilled water at 4 C until a negative test for sulfate ion was obtained and the protein started to come out of solution. The solution was then lyophilized. The recovered β-lactoglobulin, weighing 7 g, was approximately 50% of the theoretical yield.

(b) The concentrated whey was dialyzed against distilled water at 4 C until a negative test for chloride ion was obtained and incipient precipitation of the protein occurred. The solution was then lyophilized and approximately 8.5 g of β-lactoglobulin recovered.

Specific rotation. The specific rotation, $[\alpha]$, of β-lactoglobulin preparations was measured with a Rudolf Model 62 polarimeter. A Bausch and Lomb monochromator equipped with a mercury lamp was used for the light source. The high intensity spectral line at 575 mμ produced by this source was used for the measurements. The proteins were dissolved in pH 4.7 acetate buffer and placed in a 6.2-cm tube for analysis. The specific rotation, $[\alpha]$, was calculated by the equation $[\alpha] = 100\ \alpha/CL$, where α is the measured rotation in degrees, L is the length of the light path of the solution in decimeters, and C is the concentration of the protein in grams per 100 ml of solution.

Intrinsic viscosity. The intrinsic viscosity of β-lactoglobuln in pH 4.75 acetate buffer was measured at 25 C. An Ostwald capillary type viscometer, with a delivery time for 5 ml water of 65.7 sec, was used. The fluidity, ϕ, or $1/\eta_{soln.}$ was determined in the concentration range 1.5 to 0.5 g/100 ml solution. A straight line was obtained when the fluidity was plotted against the concentration, and this extrapolated to 1 at zero concentration. Data taken from the drawn line were used to derive values of the specific viscosity, η_{sp}, ($\eta_{sp} = \eta_{soln}/\eta_{buffer} - 1$) and η_{sp}/C was plotted versus C, where C is the concentration in g/100 ml of solution. Data derived in this manner plotted a straight line which, when extrapolated to zero concentration, gave the intrinsic viscosity $[\eta]$.

Free boundary electrophoresis. Free boundary electrophoretic analysis was made with a Beckman model H electrophoresis apparatus. The β-lactoglobulins, at a concentration of about 1%, were dissolved in veronal buffer and dialyzed with stirring against the buffer for 16 hr or longer before electrophoresis.

Polyacrylamide gel electrophoresis. Electrophoresis on polyacrylamide gel (PAG) was done, using Canalco apparatus according to the manufacturer's directions and their Standard Separating Gel, pH 9.5.

Negative pressure dialysis. A long-stem 3-inch glass funnel was fitted with a rubber stopper of the proper size to fit a 1- or 2-liter side-arm Erlenmeyer suction flask. Approximately one meter length of 8/32-inch dialysis tubing was attached to the stem of the funnel. The dialysis tubing was secured to the funnel stem by a 2-3-cm length of Tygon tubing. The other end of the dialysis tubing was tied in a knot, and the tubing filled with the solution to be concentrated. The filled dialysis tubing was coiled inside the Erlenmeyer flask and the latter attached to a vacuum source through its side-arm. The remainder of the solution was placed in an appropriate sized volumetric flask, secured in a vertical position upside down over the funnel. The whey constituents in true solution, such as the lactose, salts, and smaller peptides pass through the dialysis tub-

ing under the pressure difference which approaches one atmosphere. This procedure has been called negative pressure dialysis by its developer, Porath (12).

Results

The solubility of individual serum proteins in solutions with TCA concentrations ranging up to 4% is shown in Figure 1. β-Lactoglobulin is considerably more resistant to the precipitating action of TCA than the other proteins investigated. Electrophoretic patterns in PAG of proteins remaining in whey containing different amounts of TCA are shown in Figure 2. Higher than normal concentrations of protein were used to detect small concentrations of any protein not precipitated by TCA. In whey containing 3% TCA, only β-lactoglobulin is detectable.

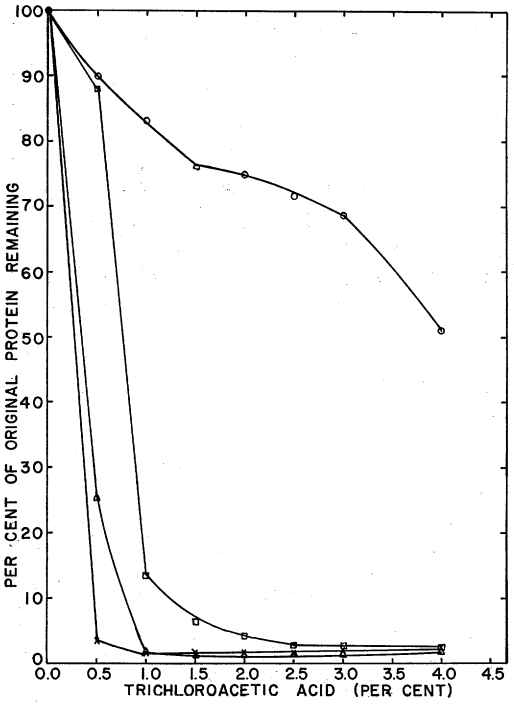

FIG. 1. Solubility of individual whey proteins at different concentrations of TCA. The proteins were initially dissolved in sodium phosphate buffer, pH 7.10.

○——○ 0.66 g/100 ml of β-lactoglobulin
□——□ .71 g/100 ml of bovine serum albumin
△——△ .60 g/100 ml of α-lactalbumin
×——× .73 g/100 ml of bovine γ-globulin

β-Lactoglobulin isolated by specific precipitating action of TCA had the physical properties listed in Table 1. Corresponding values characterizing 3 × crystallized commercial preparations of salt fractionated β-lactoglobulin are shown for comparison.

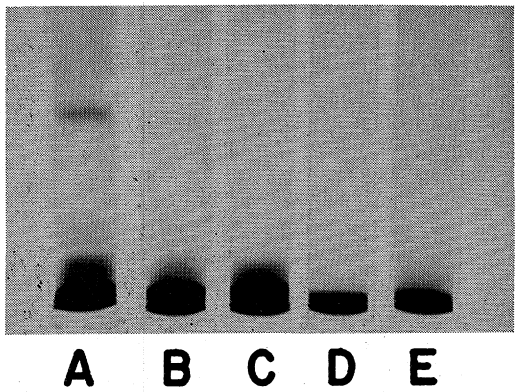

FIG. 2. PAG electrophoretic patterns of the proteins remaining in pH 4.7 whey treated with (A) 1% TCA; (B) 2% TCA; (C) 3% TCA; (D) 3% TCA; (E) 3 × crystallized β-lactoglobulin (a commercial preparation). (A), (B), and (C) contained 350 μg of protein, and (D) and (E) 150 μg protein.

TABLE 1
Some properties of β-lactoglobulin

	Preparation	
	TCA	3 × crystallized
Specific rotation at pH 4.75 and 575 mμ	−27.8	−26.7
Intrinsic vicosity at pH 4.75	4.1	3.8
Mobility × 10^{5}[a] cm^2 $volt^{-1}$ sec^{-1} at pH 8.6	−5.64	−5.69

[a] Average of two determinations.

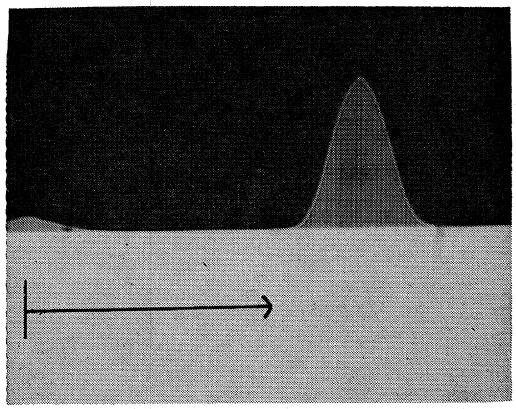

FIG. 3. Tiselius electrophoretic pattern of 1% solution of β-lactoglobulin in veronal buffer, pH 8.59, Γ/2 = 0.1. Time, 417 min. Field strength: 3.70 v/cm.

An untouched photograph of a free boundary electrophoretic pattern of β-lactoglobulin prepared by use of TCA is shown in Figure 3.

Discussion

Our observed effects of variations in concentration of TCA on bovine blood and milk whey proteins are similar to results obtained by Gofman and Sayanova (4) in their study of the precipitation of seed proteins by TCA.

A number of investigators have studied the alcohol solubility of proteins precipitated by TCA (2, 3, 5, 8). Schwert (13) demonstrated that serum albumin could be separated from the blood globulins by solubilizing the albumin-TCA precipitate in an alcoholic solution and centrifuging off the insoluble globulin TCA complexes. In this work he demonstrated that, after removal of TCA from the serum albumin, the recovered protein retained the same physical properties as serum albumin isolated by classic procedures.

In milk whey, β-lactoglobulin is the protein most resistant to the precipitating action of TCA and, therefore, the complex it forms with TCA remains in solution after all other whey proteins are precipitated. Removal of the bound TCA allows recovery of the apparently unaltered protein.

The measurement of specific rotation forms the basis for a relatively sensitive test for denaturation, since the characteristic values for the native proteins increase on denaturation. The specific rotation of β-lactoglobulin listed in the literature (7) is −30.4 at 588 mμ. When β-lactoglobulin prepared by either ammonium sulfate precipitation or by the TCA procedure was precipitated again by TCA, the specific rotation of the recovered β-lactoglobulin was decreased by about three degrees. These data suggest that TCA reacts preferentially with protein molecules having an open or unfolded structure. The specific rotations of bovine serum albumin and α-lactalbumin are −49 and −60 (7). These specific rotations are large compared to that of β-lactoglobulin and are consistent with these proteins, being much more completely precipitated from solution at a lower TCA concentration than is β-lactoglobulin.

Our demonstration that crystallizable proteins may be heterogeneous in respect to the effect of bound anions of a precipitating agent is also consistent with observations made during early studies of the binding of detergents by pure proteins (6, 10).

The intrinsic viscosity, $[\eta]$, of a protein depends on two factors, the shape of the protein molecule and degree of hydration (14). As a result proteins are divided into two groups by this property. Globular proteins have $[\eta]$ values of about 4.0 cc/g, whereas rod-shaped proteins such as myosin and collagen have $[\eta]$ values of 217 and 1,150 cc/g (14). Any unfolding or significant change in the hydration of the β-lactoglobulin molecule must result in a significant change in its $[\eta]$. The values of $[\eta]$ shown in Table 1 are in close agreement with each other and indicate that no significant change in hydration or shape was induced in β-lactoglobulin by the TCA at the concentrations used.

The electrophoretic mobilities of β-lactoglobulin prepared by the TCA procedure and of a commercial preparation are both higher than those reported in the literature (7). However, we have consistently obtained these values for different pure preparations of β-lactoglobulin and β-lactoglobulin in an unfractionated mixture of whey proteins.

The procedure of concentrating dilute protein solutions by negative pressure dialysis is preferable to concentration by pervaporation, in that proteins are not subject to drying out and denaturing on the walls of the dialysis tubing. Also, it can be done at any temperature down to, but not including, freezing.

The isolation of β-lactoglobulin as described in the b) part of the procedure yields a pure protein preparation in a minimum number of operations. The material dialyzed free of TCA can be crystallized by adjusting pH and dialyzing further, as described by Larson and Jenness (9). The procedure appears to selectively precipitate open or unfolded protein molecules. Whether this selectivity is related to the genetic variants of β-lactoglobulin was not determined.

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